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14. ABSTRACT The signal transducer and activator of transcription (STAT) 5b is involved in tumorigenic signaling in a number of cancers including breast cancer. However, its importance in breast cancer metastasis has not been investigated. Thus, this research aims to determine the role of STAT5b in breast cancer migration, invasion, and metastasis. We have found that STAT5b is essential for BT-549 and MDA-MB-231 migration to serum, as evidenced by the fact that knockdown of STAT5b inhibits migration in these cell lines by 60-80%. In addition, STAT5b knockdown inhibits BT-549 invasion through Matrigel. Furthermore, while these cells migrate well to vitronectin, this migration is inhibited in the absence of STAT5b. Therefore, STAT5b is important for migration and invasion of breast cancer cells and may be involved in adhesion of these cells to vitronectin. Further elucidating the mechanism by which STAT5b promotes migration and invasion of breast cancer cells may lead to more effective treatments for preventing and treating metastasis.					
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Introduction

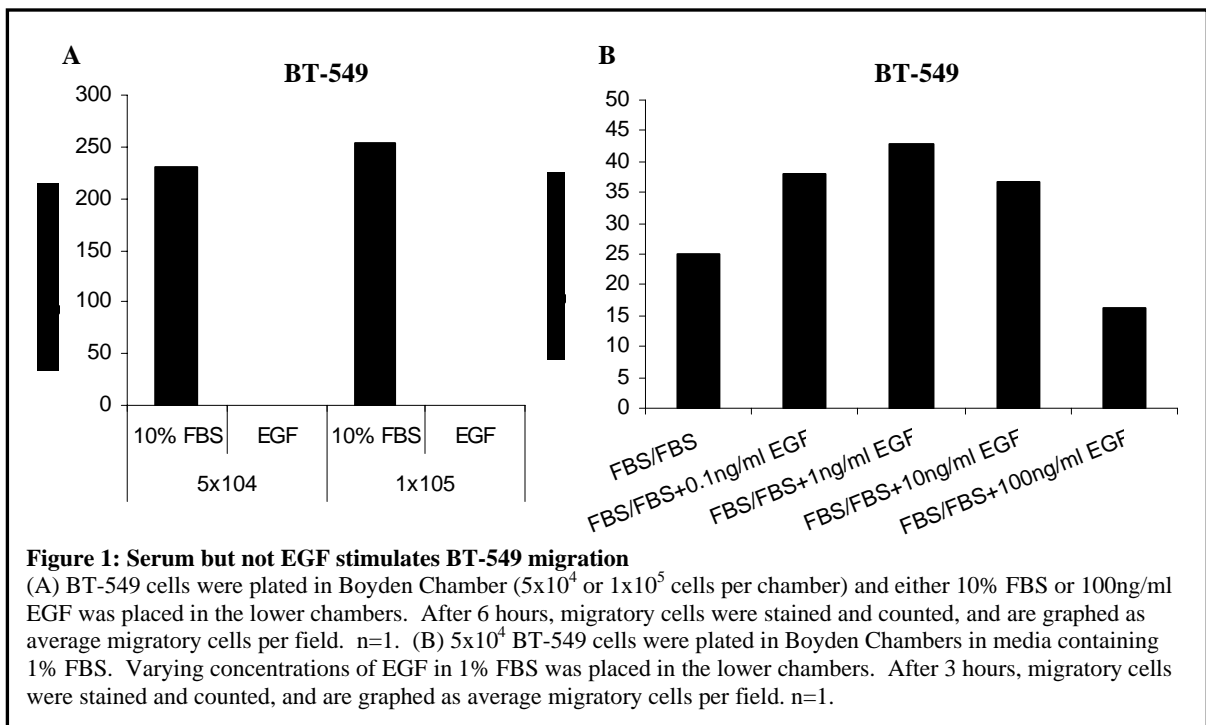
Signal transducer and activator of transcription (STAT) 5b is a transcription factor which promotes breast cancer proliferation and survival [1,2]. In these cells, STAT5b is activated downstream of the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor whose overexpression has been correlated to decreased patient survival and increased metastasis [3]. While STAT5b has been shown to be essential for invasion of metastatic prostate cancer cells, its role in breast cancer metastasis has not been examined [4]. The purpose of this research is to determine the importance of STAT5b for breast cancer cell migration, invasion, and metastasis. Ascertaining the contribution of STAT5b to breast cancer metastasis may aid in developing better targeted therapeutics for the prevention and treatment of breast cancer metastasis.

Body

Task 1: Determine the role of EGF-induced STAT5b activity in breast cancer metastasis

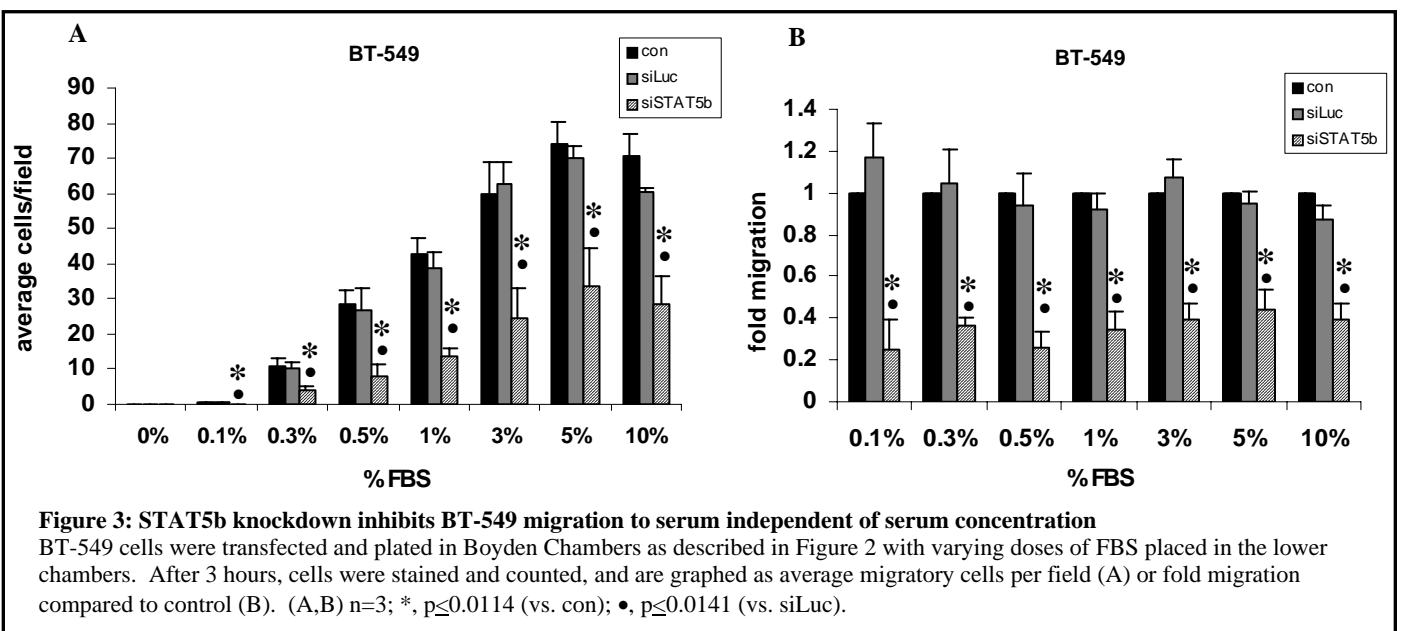
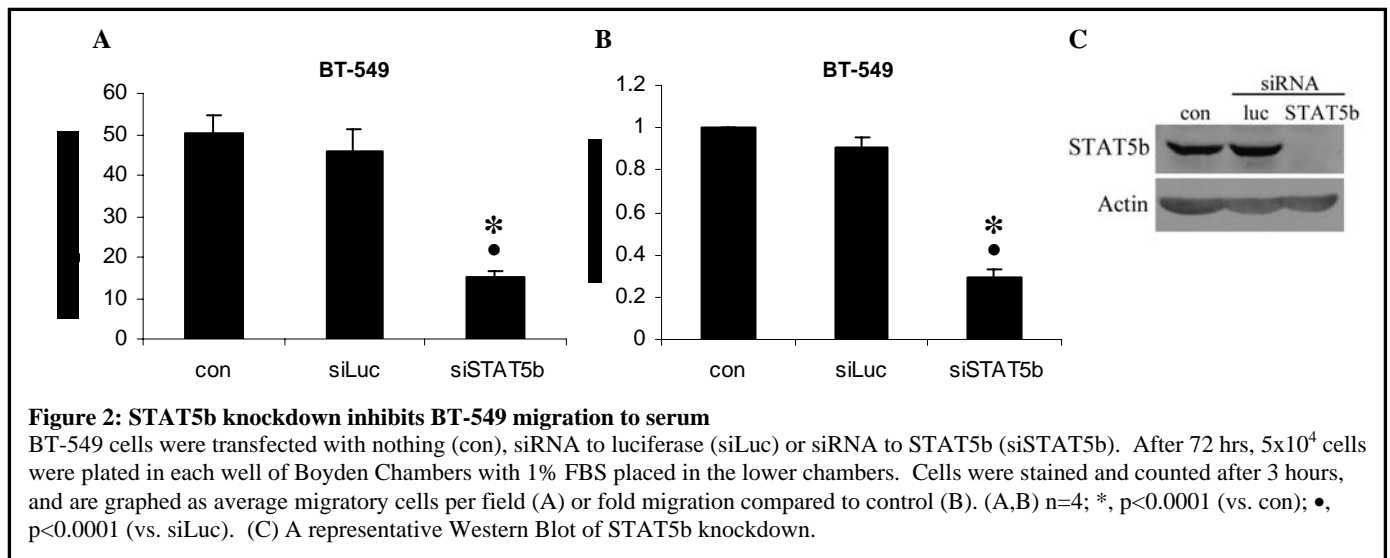
A. Investigate the effects of EGF-induced STAT5b activation on migration and invasion of breast cancer cells

Epidermal growth factor (EGF) induces migration of multiple breast cancer cell lines [5,6]. Therefore, we wanted to examine whether BT-549 breast cancer cells migrate to EGF. Surprisingly, BT-549 cells did not migrate to 100 ng/ml EGF, but exhibited robust migration to 10% FBS (Figure 1A). Since the cells were plated in serum-free media, we next tested whether the presence of serum was necessary for these cells to migrate to EGF. Furthermore, it has been shown that a 100ng/ml concentration of EGF inhibits the migration of MDA-MB-231 breast cancer cells [7]. Thus, we also tested a range of EGF concentrations. Media containing 1% FBS was placed in both the upper and lower chambers and varying concentrations of EGF was added to the lower chambers. As seen in Figure 1B, the presence of serum alone induced cell migration and the addition of 1ng/ml EGF further stimulated migration. Similar to MDA-MB-231 cells, 100 ng/ml EGF is inhibitory for BT-549 cell migration. However, because these cells migrated so efficiently to serum in a very short period of time (3 hours), our subsequent studies use serum as a chemoattractant for examining the role of STAT5b in BT-549 migration.



The original research plan proposed designing and using cell lines which stably overexpress STAT5b in order to examine the effect of STAT5b overexpression on migration and invasion of both normal breast epithelial (184A1) and breast cancer (BT-549) cells. However, our first experiment addressed whether or not STAT5b was required for BT-549 migration and invasion. We took an siRNA approach to knockdown STAT5b in the BT-549 breast cancer cells, which migrate extremely well as shown in Figure 1. In order to accurately quantitate migration, we first optimized our migration conditions, and found that plating 5×10^4 cells in the upper chambers with 1% FBS placed in the bottom chambers, and allowing the cells to migrate for 3 hours, allowed for optimal quantitation. Under these conditions, knockdown of STAT5b significantly inhibits BT-549 migration to serum by greater than 75% (Figure 2). Next, we tested whether increasing concentrations of serum could overcome the effect of STAT5b knockdown. As

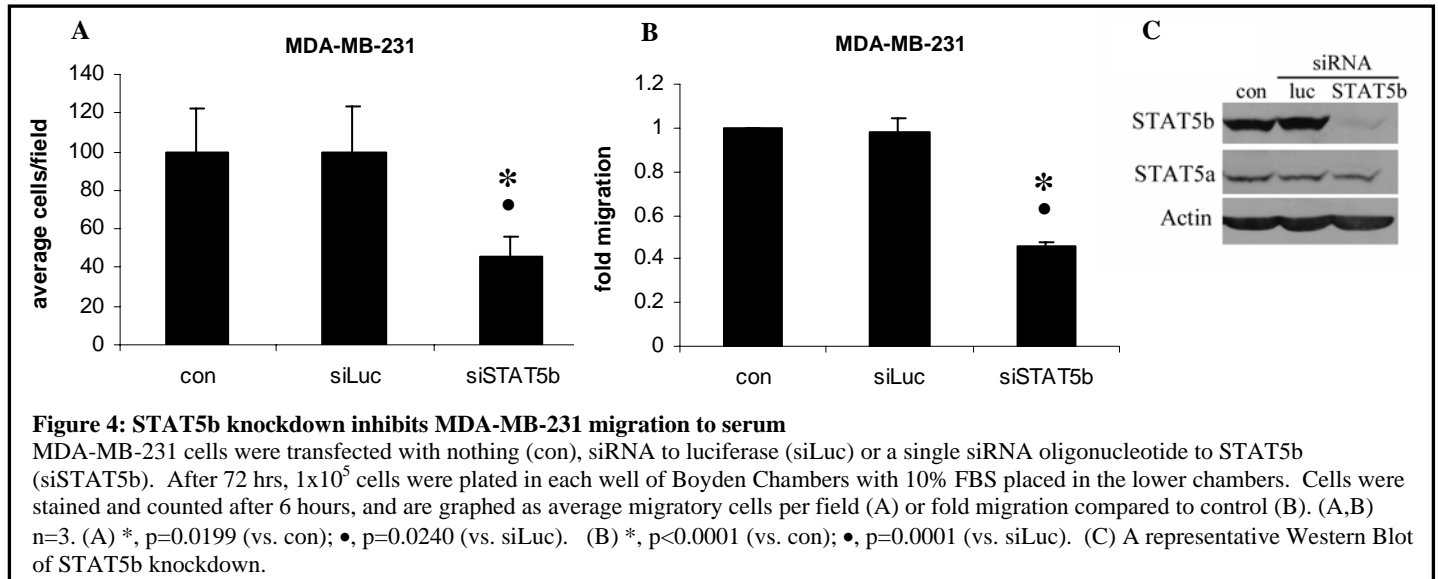
shown in Figure 3, knockdown of STAT5b decreases migration by 60-80%, regardless of serum concentration. Taken together, these results show that STAT5b is necessary for BT-549 migration to serum.



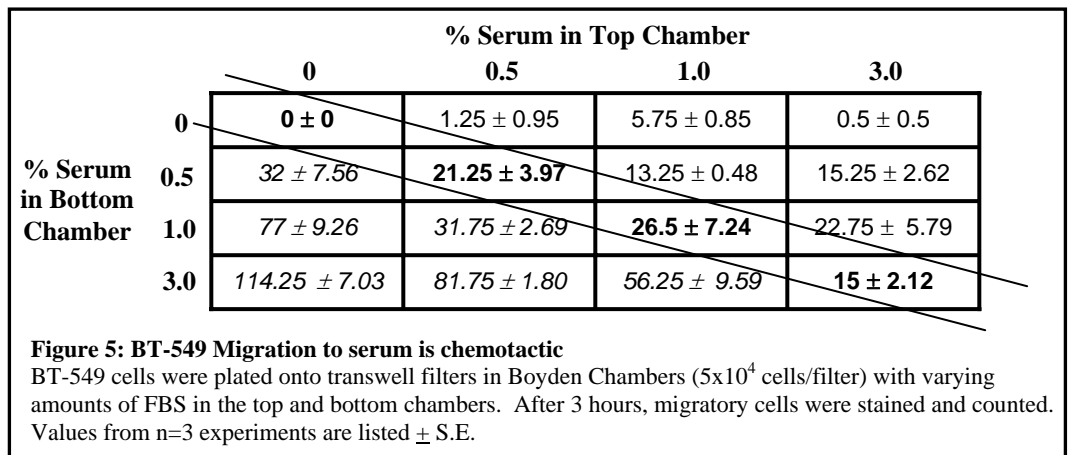
It is well established that STAT5b promotes breast cancer cell proliferation and survival. To confirm that this inhibitory effect on migration was not due to inhibition of proliferation and/or survival, we performed trypan blue assays on these cells 72 hours post-transfection. Knockdown of STAT5b does not significantly affect proliferation, survival, or adherence at 3 or 6 hours (data not shown). Therefore, we conclude that the inhibition of migration is not due to a secondary effect of STAT5b knockdown, but rather that STAT5b plays an important role in the migratory pathway.

To ensure that this migratory function of STAT5b is not unique to BT-549 cells, we examined another highly invasive breast cancer cell line, MDA-MB-231. Unlike BT-549 cells, which only express STAT5b, MDA-MB-231 cells express both STAT5b and its highly homologous family member STAT5a. STAT5b is pro-proliferative in breast cancer, whereas STAT5a is pro-differentiating and suppresses invasion [8,9]. Thus, our studies center on STAT5b. The STAT5b siRNA used for the BT-549 studies is

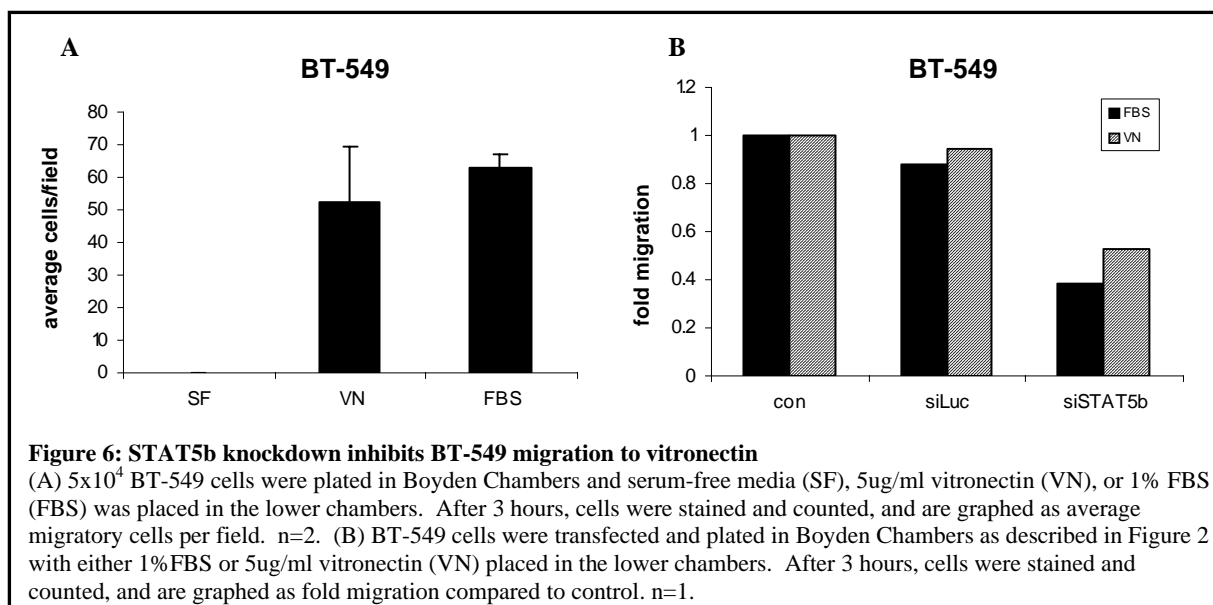
a SMARTpool of four individual oligonucleotides (Dharmacon). Unfortunately, this pool is not specific for STAT5b in cell lines expressing both proteins (data not shown). Upon further investigation, we determined that one of the oligos from this pool efficiently knocks down STAT5b but does not affect STAT5a protein expression (Figure 4C), and therefore, was used for the following MDA-MB-231 studies. In MDA-MB-231 cells, specific knockdown of STAT5b inhibits migration to serum by approximately 60% (Figure 4A,B). Trypan blue studies are currently being performed to validate that this effect of STAT5b knockdown is on migration and not inhibition of proliferation, survival, or adherence. The trypan blue studies performed in the BT-549 cells showed that STAT5b knockdown does not affect cell number or adherence over 6 hours. Therefore, we predict that the effect of STAT5b knockdown in the MDA-MB-231 cells is due to inhibition of migration, and thus, that the positive role of STAT5b in breast cancer cell migration is not cell-line specific.



Migration can be either random (chemokinesis) or directional (chemotaxis). To determine what type of migration is occurring in BT-549 cells, a checkerboard assay was performed (Figure 5). In this assay, different concentrations of serum are placed in the upper and lower chambers to form serum gradients. The amount of migration that occurs in the absence of a gradient is defined as random migration, or chemokinesis, and is indicated by the diagonal lines. Above the lines are negative gradients in which serum concentrations are higher in the upper chamber. Below the lines are positive gradients in which serum concentrations are higher in the lower chamber. If chemotaxis is occurring, cell migration should increase with increasing positive gradients. Indeed, this is what is seen in the BT-549 cells. Raising the serum concentration in the bottom chamber increases migration up to 100-fold. Therefore, we conclude that serum stimulates chemotactic movement of BT-549 cells.

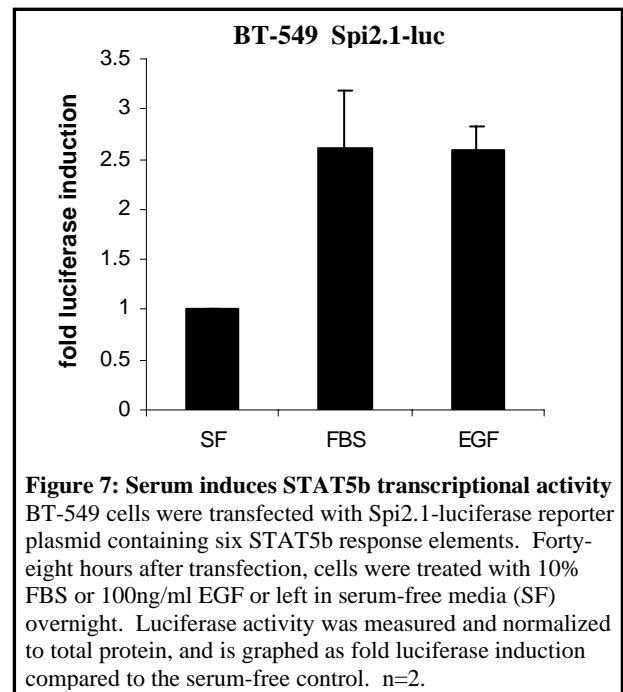


An important signaling pathway for breast cancer cell migration is integrin signaling. Integrin receptors serve as scaffolds to recruit kinases and other signaling molecules to the cytoskeleton in order to regulate cell adhesion and promote migration. The integrin $\alpha\text{v}\beta 3$ is involved in tumorigenesis in a number of cancers including breast cancer [10]. Studies have implicated EGFR/c-Src/STAT signaling in this pathway. In NIH3T3 cells, $\alpha\text{v}\beta 3$ forms a complex with EGFR, c-Src, p130Cas, and Crk, leading to phosphorylation and activation of the EGFR and c-Src [11]. Specifically, the EGFR is phosphorylated on tyrosine 845, a c-Src phosphorylation site that is important for STAT5b phosphorylation and transcriptional activity [12]. To test whether $\alpha\text{v}\beta 3$ integrin signaling promotes migration of BT-549 cells, we placed vitronectin, a known ligand for $\alpha\text{v}\beta 3$ found in serum, in the lower wells of Boyden Chambers. As Figure 6A shows, BT-549 cells migrate to vitronectin as well as they migrate to serum. Knockdown of STAT5b inhibits this migration by 50% (Figure 6B). These experiments suggest that STAT5b is a critical component of integrin-mediated migration in breast cancer cells. Furthermore, our studies indicate that STAT5b may regulate transcription of Cyr61, another ligand of $\alpha\text{v}\beta 3$ integrin, as STAT5b increases transcription from the Cyr61 promoter 2-fold (DOD preliminary data). Thus, STAT5b may be important for Cyr61 production and subsequent integrin activation.

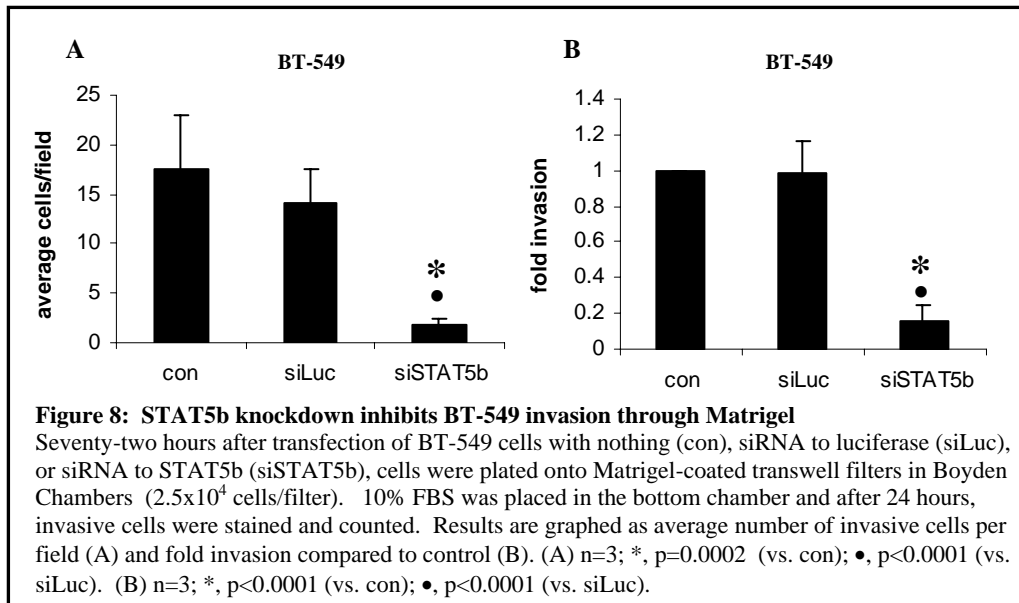


We have begun to examine the role of STAT5b transcriptional activity in breast cancer cell migration, to establish whether this function is required for its pro-migratory effect. Our initial studies show that serum stimulates transcription of the STAT5b reporter plasmid, Spi2.1 approximately 2.5-fold (Figure 7). Further studies to determine whether STAT5b is promoting breast cancer cell migration via its canonical transcriptional function, or a novel cytoplasmic activity are discussed below.

Lastly, we are also beginning to examine the role of STAT5b in breast cancer cell invasion. Knockdown of STAT5b in BT-549 cells decreases invasion through Matrigel by greater than 80% (Figure 8). This is not surprising since cells must be able to migrate in order



to invade. Trypan blue studies show this effect is not due to changes in proliferation, survival, or adherence (data not shown). Future studies will further examine this effect in BT-549 as well as MDA-MB-231 cells.



B. Determine if STAT5b is important for metastasis of breast cancer cells *in vivo*

No *in vivo* animal studies have been performed to date.

Task 2: Elucidate the mechanism by which STAT5b promotes breast cancer progression

A. Determine if STAT5b regulates transcription of tumor-secreted proteins Cyr61 and AM

B. Establish whether STAT5b is necessary for Cyr61- and AM- mediated tumor cell migration and invasion

No additional studies have been performed for Task 2 to date.

Future Studies

Future studies will be aimed at further elucidating the mechanism by which STAT5b is promoting migration of BT-549 and MDA-MB-231 breast cancer cells. Two pathways will be examined: CXCR4/SDF-1 β chemokine signaling and $\alpha v \beta 3$ integrin signaling. To begin to examine the role of CXCR4 chemokine signaling, we must first optimize the conditions necessary for observing migration to the CXCR4 ligand SDF-1 β . After this is accomplished, siRNA experiments will be performed to determine the necessity of STAT5b in this process. We will test whether CXCR4 neutralizing antibodies inhibit cell migration to serum, thus allowing us to determine if SDF-1 β is the serum component responsible for stimulating breast cancer cell migration. In addition, we will see if a constitutively active STAT5b can overcome this inhibition.

To address the integrin pathway, we will repeat the vitronectin experiments in Figure 6 in order to confirm that STAT5b inhibits migration to vitronectin. We will use neutralizing antibodies to determine if integrin signaling is responsible for the observed cell migration, and if so, whether a constitutively active STAT5b can overcome this inhibition. $\alpha v\beta 3$ can transactivate the EGFR and therefore, we will use EGFR kinase inhibitors to determine if EGFR kinase activity is necessary for migration of these cells. We can also utilize PP2, a c-Src inhibitor, to determine the role of the non-receptor tyrosine kinase c-Src in this process.

Lastly, whether the role of STAT5b in breast cancer cell migration requires its canonical transcriptional activity or a novel cytoplasmic activity will be addressed using siRNA rescue experiments. STAT5b will be knocked down and a STAT5b immune to knockdown harboring a mutation in Y699 will be re-expressed (preventing active dimer formation and subsequent nuclear translocation). The ability of this mutant to reconstitute migration will be determined. We will also treat breast cancer cells with SDF-1 β and vitronectin and look for induction of STAT5b phosphorylation and transcriptional activity. Also, if it is confirmed that STAT5b is critical for integrin-mediated migration, we will perform chromatin immunoprecipitation (ChIP) and luciferase assays to determine whether STAT5b binds to and promotes transcription from the Cyr61 promoter.

Materials and Methods

Cell culture and reagents

BT-549 and MDA-MB-231 human breast cancer cell lines were obtained from ATCC and maintained in DMEM media supplemented with 10% fetal bovine serum (Invitrogen). Recombinant human epidermal growth factor (rhEGF) was purchased from Invitrogen. STAT5b SMARTpool and luciferase duplex siRNAs were purchased from Dharmacon. Recombinant human stromal cell derived factor 1-beta (rhSDF-1beta) was obtained from Peprotech and recombinant human vitronectin (rhVN) was purchased from Upstate. STAT5a and STAT5b polyclonal antibodies were developed in our lab [13] and the monoclonal actin antibody is from Sigma. Protease inhibitor cocktail was obtained from Calbiochem, acrylamide from Bio-Rad, and prestained molecular weight standards from Sigma. All tissue culture reagents were purchased from Invitrogen and all other reagents were of reagent or molecular biological grade from Sigma.

RNAi Transfection and Boyden Chamber Assays

BT-549 and MDA-MB-231 cells were transfected with STAT5b SMARTpool siRNA (Dharmacon), the individual STAT5b SMARTpool oligo G3, or a luciferase duplex siRNA (Dharmacon) using OligofectAMINE (Invitrogen) as per manufacturer's instructions. Seventy-two hours post-transfection, cells were transferred to BD BioCoat Matrigel Control Chambers for migration or BD BioCoat Matrigel Invasion Chambers for invasion (BD Biosciences). Migration assays were performed for 3-6 hours and invasion assays for 24 hours. After this time, non-migratory/invasive cells remaining in the upper chambers were removed with cotton swabs, and the remaining cells were stained with 0.1% crystal violet in 20% EtOH. Cells were counted using a light microscope.

Immunoblotting

Protein lysates were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose (Pall Corporation). Membranes were blocked in TBST (0.15M NaCl, 0.1% Tween 20, 50mM Tris (pH 8.0)) containing 5% nonfat dry milk and incubated with primary antibodies in TBST/5% milk. Secondary antibodies were applied in TBST and were HRP-conjugated sheep anti-mouse or donkey anti-rabbit (Amersham, GE Healthcare). The enhanced chemiluminescence detection kit (Amersham, GE Healthcare) was used to detect antibody binding.

Luciferase

BT-549 cells were transfected with the Spi2.1-containing luciferase reporter plasmid using LipofectAMINE Plus (Invitrogen) according to manufacturer's directions. Forty-eight hours post-transfection, cells were treated with 10% FBS/DMEM or 100ng/ml rhEGF in 0.1% BSA/DMEM overnight. Luciferase activity was measured using a Berthold Luminometer and normalized to total protein.

Key Research Accomplishments

- STAT5b plays an integral role in the migration of breast cancer cells to serum
- Knockdown of STAT5b inhibits the migration of BT-549 cells to vitronectin, a component of serum
- Knockdown of STAT5b does not inhibit proliferation, survival, or adherence of breast cancer cells over 6 hours
- STAT5b can be specifically and efficiently knocked down in breast cancer cells which express the highly homologous STAT family member, STAT5a
- Knockdown of STAT5b inhibits BT-549 invasion through Matrigel
- Serum increase the transcriptional activity of STAT5b in an *in vitro* reporter assay

Reportable Outcomes

- Poster Presentation at the 2007 Endocrine Society Conference (Toronto, Canada):
P2-600: Role of STAT5b in Breast Cancer Cell Migration and Invasion
Teresa M. Bernaciak and Corinne M. Silva
University of Virginia
Departments of Microbiology and Medicine and the Cancer Center
- Research & Development Grant
University of Virginia School of Medicine
Feb 1, 2007 – Jan 31, 2008
\$20,000
- Submitted July 1, 2007 to NIH
RO1 CA 122562, “Targeting the Signal Transducer and Activator of Transcription 5b (STAT5b) in Breast Cancer”
PI: Corinne M. Silva

Conclusion

In conclusion, signal transducer and activator of transcription (STAT) 5b is integral for breast cancer cell migration. Knockdown of STAT5b inhibits BT-549 and MDA-MB-231 migration to serum, as well as BT-549 migration to vitronectin. This effect is due to a direct role of STAT5b in the migratory pathway and not an indirect effect on proliferation, survival, or adherence. In addition, STAT5b is integral for BT-549 invasion through Matrigel. Cell migration and invasion are necessary steps in breast cancer metastasis. There are currently no effective treatments to prevent metastasis, the major cause of breast cancer deaths, and as a result, the five-year survival rate for metastatic breast cancer is less than 10%. Our work suggests that STAT5b is pro-migratory and pro-invasive, and thus, may provide a novel target for preventing and treating breast cancer metastasis.

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